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THE ROYAL SOCIETY

The organization of mature T-cell pools

Corinne Tanchot, Henrique Veiga Fernandes and Benedita Rocha*

Institute National de la Santé et de la Recherche Médicale U345, Institut Necker, 156 rue de Vaugirard, 75015 Paris, France

To deal with exogenous pathogens the peripheral T-cell compartment requires diverse repertoires (as those of naive cells) and efficient responses, the latter dependent on the persistence of memory cells. In the present work we show that (i) naive and memory cells differ in the type of interactions required for survival and division; (ii) they are segregated into independent ecological niches; (iii) that the size of each niche is controlled by independent homeostatic mechanisms; and (iv) that naive T cells do not have intrinsic life spans, surviving in the absence of thymus output but being continuously substituted by thymus export. The independent homeostatic regulation of the naive and memory T-cell pools guarantees the maintenance of versatile and efficient repertoires throughout life as well as the persistence of the naive T-cell pool after the thymus atrophies at puberty.

Keywords: homeostasis; lymphocyte life span; diversity; lymphocyte competition; thymus output; efficiency

1. INTRODUCTION

The capacity of the peripheral immune system to deal adequately with all types of antigen challenges requires that mature T-cell repertoires are able to recognize a very broad range of antigens throughout life. This versatility is due to the thymus generation and export of naive T cells displaying a vast array of T-cell receptor diversity. Within the naive set, however, the frequency of T cells responding to each antigen is very low: to deal with antigenic challenges efficiently, these rare cells have to be activated and expanded to generate memory T cells; efficient secondary responses depend on the maintenance of a memory T-cell pool.

A peripheral immune system, always ready to respond and able to respond efficiently, thus requires the continuous presence of both naive and memory cells, but this may be subjected to constraints. The first is space. In adult mice, the total number of T cells is kept constant, a phenomenon usually referred to as homeostatic control (Freitas & Rocha 1993). Within this 'limited space' any T lymphocyte (either a naive cell generated in the thymus or a memory T cell produced by cell division in the periphery) can only survive if another resident T cell dies. If recent thymus migrants have a survival advantage over resident T cells, their preferential incorporation into peripheral pools might lead to the extinction of previously generated memory responses. Alternatively, if dividing T cells survive better than thymus migrants, memory clones may accumulate, resulting in a gradual reduction in naive T cells and lesser repertoire diversity. The presence of a 'limited space' raises the paradox of how to reconcile versatility (dependent on the naive T-cell survival) with efficiency (dependent on memory survival). The other limitation to the maintenance of the peripheral T-cell pool is the possible extinction of naive T-cell production in the thymus, when this organ involutes after puberty.

*Author for correspondence (rocha@necker.fr).

We studied the organization of the peripheral T-cell pools by a double approach. First, we used a very simplified experimental system, in which we analysed the behaviour of a single clone of T cells *in vivo*. This TcR clone was well-characterized previously: we knew its specificity and the type of interactions that the clonal T-cell receptor (TCR) had with the major histocompatibility complex (MHC). We used the knowledge we had obtained from this clone to study the characteristics of polyclonal T-lymphocyte populations from a normal mouse.

2. NAIVE AND MEMORY T CELLS

The single clone was obtained from female mice deficient in the recombinase gene Rag2 (Kisielow *et al.* 1988), unable to rearrange endogenous T-cell receptors and expressing a single transgenic TCR, specific for the male antigen, and restricted to the H-2D^b MHC class I molecule (Shinkai *et al.* 1992). In the female mouse, these cells can be considered a bona fide population of naive cells. The male antigen is absent and there is no cross-reactivity with environment antigens since these cells neither divide (Von Boehmer & Hafen 1993) nor express messenger RNA (mRNA) for cytokines (Tanchot *et al.* 1997). These cells are also CD44$^-$, a cell-surface marker useful to identify naive T cells.

After *in vivo* immunization with male cells these Tg cells become activated, expand, eliminate the male cells and persist as memory T cells. When Tg memory cells are studied, it is found that they are all CD44$^+$, express mRNA for γ-interferon and IL-2, and divide slowly (Tanchot *et al.* 1997).

The CD8$^+$ T-cell pool from normal B6 mice also contains CD44$^-$ and CD44$^+$ T cells. As far as we could test, these CD44$^-$ and CD44$^+$ normal T cells behave like naive and memory Tg cells respectively (figure 1). CD44$^-$ cells do not divide or express mRNA for cytokines. CD44$^+$ cells divide at the same rate (Tanchot *et al.* 1997;

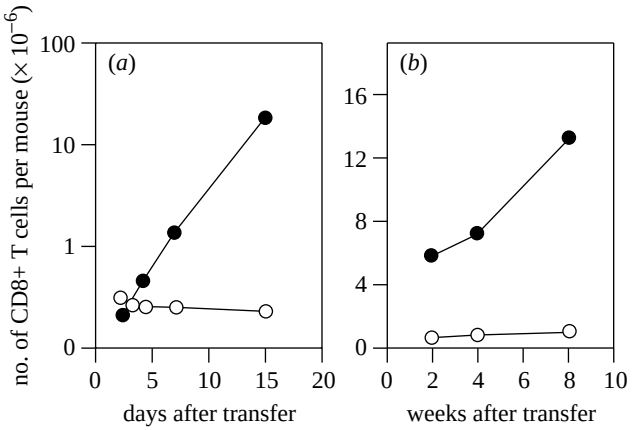

Figure 1. Memory but not naive T cells are able to proliferate in the absence of stimulation by the nominal antigen. The same number (0.5×10^6) of CD44$^-$ and CD44$^+$ CD8$^+$ T cells from C57Bl/6 mice were cotransferred to T-cell deficient, female, syngeneic hosts. Results represent the number of CD44$^-$ (open symbols) and CD44$^+$ CD8$^+$ cells (filled symbols) recovered from these hosts at different times after transfer. (*a*) Injected cells were monoclonal male-specific TcR-Tg cells (Tg cells) (Th1.1 CD44$^-$ naive and Thy 1.2 CD44$^+$ memory). (*b*) Injected cells were polyclonal CD8$^+$ T cells from a C57Bl/6 normal mouse.

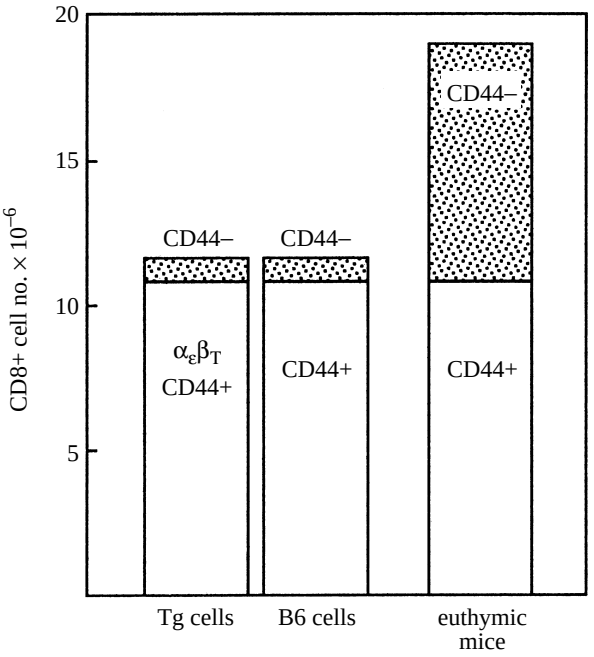

Figure 2. Peripheral expansion of memory cells reconstitutes the memory T-cell niche. The results compare the number and phenotype of CD8$^+$ T cells recovered from female age-matched syngeneic C57Bl/6 mice (left and middle) that were T-cell deficient and injected two months previously with lymph node peripheral TcR-Tg (left) and non-Tg (middle) CD8$^+$ T cells with normal euthymic mice (right). (The data are taken from Tanchot & Rocha (1995).)

Tanchot & Rocha 1995) and express cytokine mRNA at same level as memory CD8$^+$ Tg cells (Tanchot *et al.* 1997).

We recently extended this analysis to the characterization of a panel of other cell-surface markers: LFA1, L-selectin, CD69, IL-2Rα, β, γ-chains, CD5, TcR, $CD_3\varepsilon$ as well as the different isoforms of CD45. We found the same distribution of surface markers in naive Tg and CD44$^-$ normal cells, while memory Tg populations had the same phenotype as CD44$^+$ normal cells (Guillaume and B. Rocha, unpublished data). All our evidence suggests, therefore, that naive and memory CD8$^+$ T cells from normal mice (identified by their level of CD44 expression) have the same characteristics as Tg cells.

3. THE DIFFERENT INTERACTIONS REQUIRED FOR NAIVE AND MEMORY T-CELL SURVIVAL

We adoptively transferred naive and memory Tg cells, to male or female mice expressing H-2D^b, or to mice deficient in different MHC alleles. We found that survival of naive Tg cells required continuous interaction with the TcR-Tg H-2D^b MHC-restricting element, since these cells died when transferred to mice lacking H-2D^b. In the same adoptive transfer, memory Tg cells survived in the absence of the MHC-restricting element but decayed after transfer into $\beta_2 m \times D^b$ deficient mice (used as hosts expressing little MHC class I). CD8$^+$ T lymphocytes from normal mice behaved similarly: 90% disappeared one week after transfer into mice lacking MHC class I glycoproteins (Tanchot *et al.* 1997).

It thus appears that mature naive cells, once having left the thymus, must continuously recognize MHC to survive in the periphery, a process similar to thymus positive selection. The required TcR ligation is MHC restricted and probably maintains bcl-2 expression, as reduced levels are found when interactions with MHC are discontinued (Kirberg *et al.* 1997). It is also clear that antigen-experienced cells follow different rules: these cells appear less particular regarding the type of MHC molecule they interact with.

The different interactions involved in the survival of naive and memory cells suggests that these cells may seek different ecological niches.

4. THE DIFFERENT INTERACTIONS REQUIRED FOR NAIVE AND MEMORY T-CELL DIVISION

Naive Tg cells require antigen stimulation to divide (Tanchot *et al.* 1997), while memory T cells can expand in the absence of nominal antigen stimulation. This difference in behaviour is obvious when naive and memory Tg cells are cotransferred to irradiated female mice: in these female hosts only CD44$^+$ Tg memory T cells expand. The same phenomenon is observed when CD44$^-$ and CD44$^+$CD8$^+$ T cells from normal B6 mice are cotransferred to the same hosts: in the absence of intentional antigen stimulation, the number of CD44$^-$ cells does not change with time, while the CD44$^+$ set expands (figure 2). The different interactions involved in the expansion of naive and memory cells also support the notion that these cells may belong to different ecological niches.

5. NAIVE AND MEMORY T CELLS BELONG TO DIFFERENT ECOLOGICAL NICHES THAT ARE REGULATED BY INDEPENDENT HOMEOSTATIC MECHANISMS

To study how the size of the naive T-cell pool was regulated we studied Tg mice crossed into the Rag 2-deficient background. These mice can produce only naive CD8$^+$

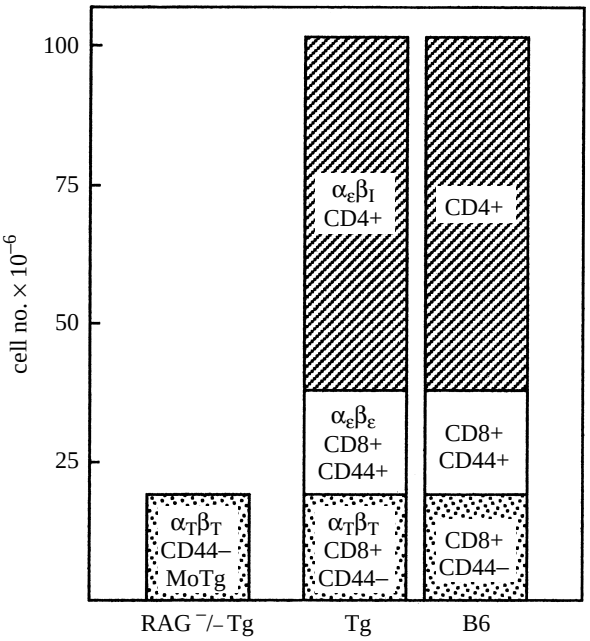

Figure 3. Thymus export of naive cells reconstitutes the naive T-cell niche. The results show the number and phenotype of peripheral T cells recovered from different female litter mates that express a transgenic T-cell receptor specific for the male antigen (left and middle), lack the recombinase gene Rag2 (left) and T cells from non-Tg mice (right). (The data are taken from Tanchot & Rocha (1995).)

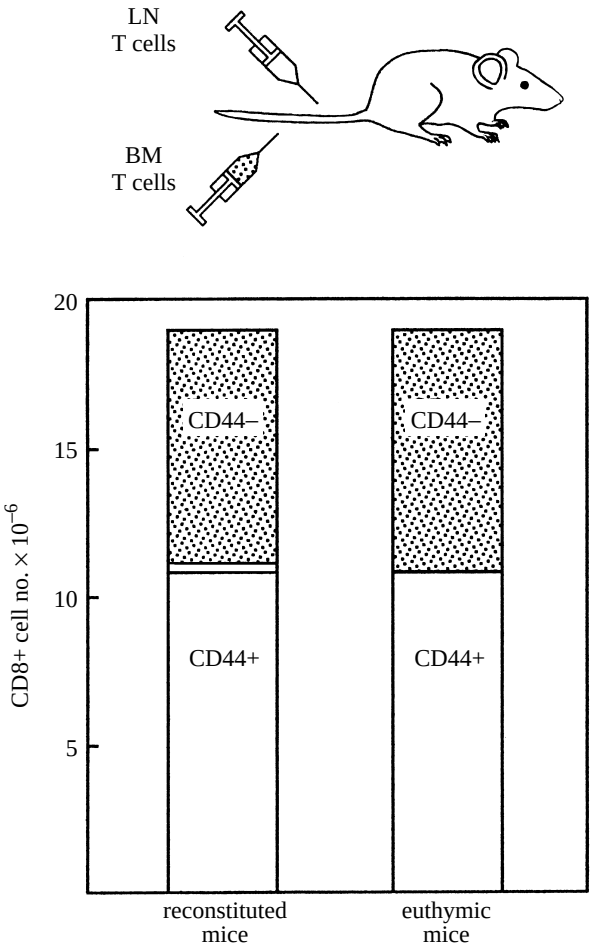

Figure 4. Reconstitution of a 'full' peripheral compartment requires thymus output of naive T cells and peripheral expansion of memory T cells. The results show the number and phenotype of $CD8^+$ T cells recovered from female age-matched syngeneic C57Bl/6 mice. Left, T-cell-deficient mice that were injected with lymph node (LN) Ly 5.1 T cells (open bar) and bone marrow (BM) Ly 5.2 cells (dotted bar). The $CD44^+$ cells are of lymph node origin, while the $CD44^-$ cells are derived from the bone marrow. Right, euthymic mouse. (The data are taken from Tanchot & Rocha (1995).)

cells. Due to the lack of recombinase, B cells, $CD4^+$ and memory $CD8^+CD44^+$ cells are absent. There is a lot of 'empty space' available. The peripheral T-cell pool is, however, very small as the number of naive lymphocytes does not increase despite the available 'space': it is the same as found in Tg $Rag^+/_+$-litter mates, and of similar size to the $CD44^-$ T-cell pool of normal mice (figure 3). These results indicate that the continuous thymus output of naive $CD44^-$ cells (unable to expand) only reconstitutes the naive Tg niche. The total size of the naive T-cell pool is thus regulated by an homeostatic mechanism(s), independently of the number of other T cells present (Tanchot & Rocha 1995).

To study the regulation of the size of the memory T-cell compartment, we generated mice in which only memory T cells were present. We injected memory T cells into mice without a thymus. Athymic mice injected with different numbers of T cells (from 10^2 to 10^7) reconstitute the periphery to similar levels (Rocha *et al.* 1989), but this peripheral expansion only reconstitutes the memory T-cell pool. The number of memory cells generated is the same as that present in an euthymic mice, i.e. half of the peripheral T-cell pool (figure 4) (Rocha *et al.* 1989). These results suggest that the total number of memory T lymphocytes is homeostatically regulated, independently of that of naive T cells. Therefore, the reconstitution of the total size of the peripheral T-cell pools requires both thymic output of naive cells and peripheral expansion of memory T cells. This is shown in T-cell-deficient hosts, injected simultaneously with peripheral T lymphocytes and bone marrow (BM) cells: the memory pool is reconstituted by the expansion of memory T cells of lymph node origin, within the first month after cell transfer. Thymus export of naive T cells of donor BM origin starts thereafter. The input of large numbers of naive thymus migrants does not modify the total number of resident memory cells: the T-cell pool doubles in size, to accommodate the newly formed naive cells migrating from the thymus. A full-size T-cell compartment is generated, where the total number of memory cells and naive cells is the same as in non-manipulated mice (figure 4) (Tanchot & Rocha 1995).

These results illustrate how the organization of the peripheral T-cell pool solves the 'versatility versus efficiency' paradox by the division of the peripheral T-cell pool into two compartments of equivalent size—a compartment of naive cells and a compartment of memory T cells—with the size of each compartment being regulated independently. The notion that memory and naive T cells do not compete for the same niches is also supported by studies of the substitution of resident T lymphocytes by thymus migrants (Tanchot & Rocha 1997). It was found that thymus output was unaffected by

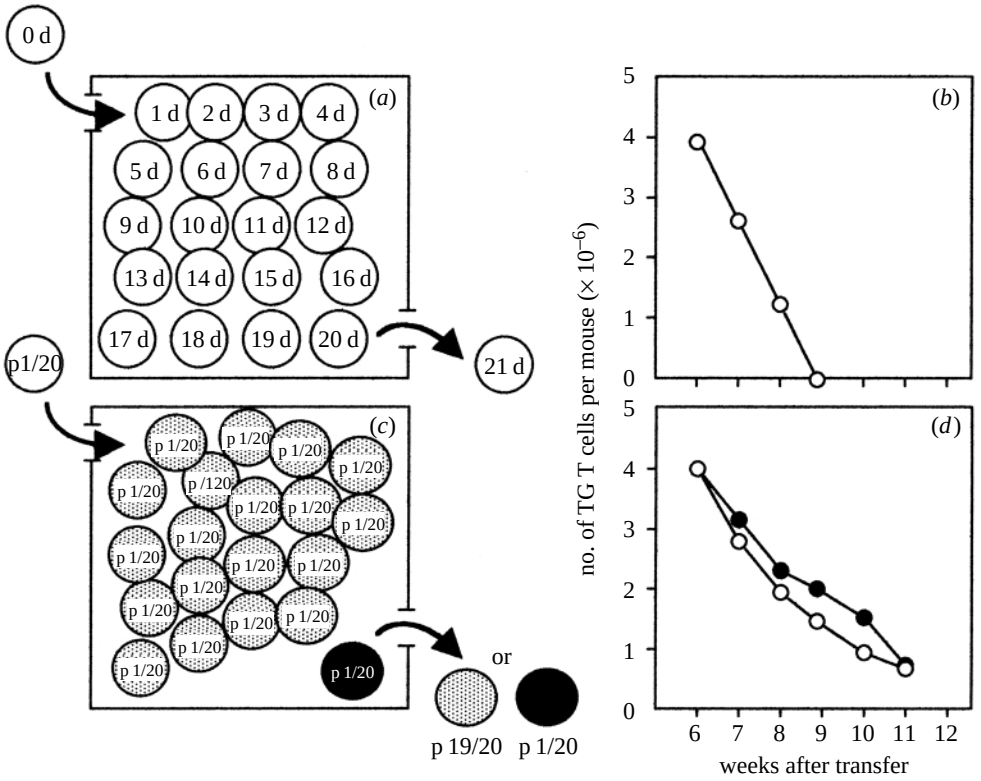

Figure 5. The two hypotheses explaining the substitution of naive resident cells by thymus migrants. (*a*,*c*) represent the periphery, where 20 elements are present, each representing one cell. (*a*) Cell substitution is age dependent. Each day the younger cells (thymus migrants) are incorporated in the periphery and the older cells die. (*b*) Linear decay according to the formula $X_t = X_0(1-at)$ (X_t, absolute number of resident Tg cells as a function of time; X_0, absolute number of resident Tg at time 0; *a*, the ratio between the number of thymus migrants (1×10^6 d^{-1}) and the total number of resident naive CD8$^+$ cells (20×10^6) T cells; *t*, time in days). (*c*) Substitution is cell age independent: a large clone (19 elements) is represented in grey and a small clone (one element) in black, with each element representing a cell (1/20) to be replaced by a thymus migrant. The probability of replacing a grey cell will be 19 times higher than that for replacing a dark cell. (*d*) Open symbols, exponential decay, calculated according to the formula $X(t) = X_0(1-a)^t$; closed symbols, experimental decay of Tg-resident T cells.

the presence of resident T cells, and that thymus migrants were unable to dislodge resident memory T cells (Tanchot & Rocha 1997).

6. THE DISTRIBUTION OF NAIVE AND MEMORY CELLS INSIDE THEIR OWN NICHES

The existence of independent niches for naive and memory cells implies that memory T-cell repertoires are unaffected by the output of naive T cells from the thymus. Conversely, successive antigen encounters and generation of memory T cells will not necessarily lead to the disappearance of the naive T-cell pool. These rules are, however, only the first degree of complexity in the organization of peripheral repertoires. The second degree, and at least of equal importance, is the definition of the rules that condition survival of different T-cell specificity within each niche.

It is as yet unknown how the survival of resident memory T cells is conditioned by the generation of new memory T cells. How the survival of resident naive cells is affected by thymus output, however, has already been evaluated.

7. THE MAINTENANCE OF THE NAIVE T-CELL POOL

The mechanisms ensuring the maintenance of naive T cells in adults have been unclear. If the thymus undergoes atrophy during puberty, and naive T cells have a finite and relatively short life span, should not all naive cells eventually disappear? Data on naive T-cell life spans are contradictory: these cells were shown to have very long life spans or indefinite survival in the periphery when transferred to thymusless mice whereas, in euthymic mice, resident naive cells were relatively short lived, with an average of 50% being renewed by thymus migrants every two weeks (Von Boehmer & Hafen 1993; Tough & Sprent 1994). Recent data on the renewal of a clone of resident peripheral CD8$^+$ TcR-Tg naive cells by polyclonal thymus migrants from normal mice clarified this issue (Tanchot & Rocha 1997). It was found that recent migrants replaced resident Tg naive cells, but that this substitution was age independent. The term 'age independent' makes all the difference.

Indeed, there are two possible explanations for the replacement of peripheral T cells: survival is either cell age dependent or age independent (figure 5*a*,*c*). In the first hypothesis, T cells have a finite life span being committed to die at a certain age. Each day the younger cells (recent thymus migrants) are incorporated into the periphery, while the oldest resident cells die. In this scenario, the substitution of resident cells is linear (figure 5*b*). We found, however, that resident T-cell decay was exponential, a situation only compatible with age-independent substitution (figure 5*d*). In this latter case,

T lymphocytes do not have intrinsic life spans, but can potentially survive indefinitely in the absence of input from the thymus. Resident naive cells can be replaced when naive thymus migrants are competing for the same niche, but each cell in the periphery or in the cohort of thymus migrants has the same survival probability. Life spans can thus only be defined as this relativistic probability of survival.

This type of substitution ensures that the naive T-cell pool will persist after thymus atrophy. In the absence of thymus output, naive T-cell decay will be conditioned solely by successive antigen stimulation that may recruit naive T cells into the memory T-cell pool.

Age-independent substitution provides indirect information on the nature of the interactions ensuring naive T-cell survival. We found that TcR fine specificity has little influence on the survival rate of naive T cells: each cell within a clone of Tg cells or within polyclonal T-cell populations expressing very diverse TcRs had a similar survival probability. In this respect, naive T-cell survival also mimics thymus positive selection, which is probably more strongly influenced by MHC recognition than by peptide recognition, as shown by the selection of T lymphocytes with diverse receptors by a single MHC–peptide ligands (Kisielow & Miazek 1995).

Age-independent random substitution also influences relative clone size distribution imposing repertoire diversity, as illustrated in figure 5*c*, where a large clone is represented by 19 grey elements and a small clone by one dark element. Random substitution will be equivalent to blind removal of an element. Although each element has the same probability of being removed (one in 20), the probability that the element removed belongs to a large clone (i.e. grey) will be 19 times higher than that of removing the dark element. Decay curves following exponential laws will lead to rapid contraction of large clones and to relatively prolonged survival of small clones, thereby determining a peripheral T-cell repertoire composed of many small clones. In normal mice, this mechanism of diversification of naive T-cell repertoires probably occurs during thymus 'positive selection'. It takes two weeks for $CD4^+CD8^+$ immature thymocytes to generate thymus migrants (Rooke *et al.* 1997; Pénit & Ezine 1989) and, during this relatively long period, selection of Tg cells increases when these cells represent only a minority of immature thymocytes (Huesmann *et al.* 1991).

8. THE MEMORY T-CELL POOL

Like naive T cells, memory T cells do not require recognition of their cognate ligand (antigen) to survive (Hou *et al.* 1994; Lau *et al.* 1994; Mullbacher 1994), although some type of interactions with self-MHC may be required. In contrast to naive T cells, however, memory cells can divide, sometimes extensively, in an antigen-independent fashion.

In normal mice, at least a fraction of memory cells are cycling. The rate of division *in vivo* can be increased by the injection of growth factors such as IL-2, IL-12 and α-interferons (Hou *et al.* 1994; Lau *et al.* 1994; Mullbacher 1994; Sprent *et al.* 1997), or during immunization. Activation of memory T lymphocytes of unrelated T-cell specificity is then observed, and up to 70% of $CD8^+$ T cells can be recruited into the cell cycle (Doherty *et al.* 1996). This bystander activation is probably a consequence of growth factor production *in vivo*.

The rate of memory-cell division is also increased after partial T-cell depletion: residual memory cells expand, a phenomenon responsible for early reconstitution of the peripheral T-cell pools in irradiated recipients (reviewed in Mackall *et al.* 1997), or in secondary immunodeficiencies such as AIDS.

9. MEMORY T-CELL SURVIVAL IS NOT SOLELY CONDITIONED BY TCR-PEPTIDE RECOGNITION

Perhaps the most straightforward way of phrasing the problem of memory T-cell survival is to equate it to the old problem of the involvement of peptide in thymus positive selection. To what extent is peptide recognition required for memory T-cell survival? Two extreme alternatives, and all intermediate situations, can be envisaged.

In the first, survival of memory cells depends on the recognition of a very restricted range of peptides. Memory cells divide in environments where naive cells do not divide because memory T cells are able to recognize a larger range of peptides than naive cells. In this circumstance, it can be said that memory is maintained by cross-reactive antigens, although this cross-reactivity is an acquired property of memory cells. It must be noted that if maintenance of memory is conditioned by cross-reactivity, (i) it may be hazardous, as it depends on the possibility that memory T cells have joint specificity for other antigens (self-antigens or environment antigens to which the immune system was frequently primed); (ii) T lymphocytes may be able to change their range of peptide recognition (antigen specificity) during the course of T-cell activation. In this scenario, how is the frequent emergence of self-reactivity avoided?

In the second alternative, as in the case for naive T cells, memory T-cell survival may occur when a very broad range of peptides is presented by the MHC, and may rely mainly on MHC recognition. Antigen recognition by naive and memory cells would be similar, i.e. the reduced activation thresholds of memory T cells would not interfere substantially with the number of peptides able to trigger their TcR, but would ensure that signals associated with MHC recognition should result in cell division rather than just survival (as is the case in naive cells). The rate of division would be increased by environment growth factors. If memory is maintained mostly by MHC interactions, (i) the survival of memory cells will not reflect the antigen environment, as memory cells generated in response to different antigens are equally likely to persist; (ii) the distribution of memory T-cell specificity in the peripheral pools will be expected to follow rules similar to those found in the naive T-cell pools. After the elimination of antigen, age-independent substitution of memory T cells with other memory cells would lead to the rapid contraction of large clones (as seen in the contraction phase of most immune responses) and to the relative prolonged survival of small clones of memory cells, determining a memory T-cell repertoire composed of many T-cell clones of small size, i.e. imposing repertoire diversity; and (iii) TcR triggering of memory cells will likely be a phenomenon very different from non-antigen-dependent expansion.

10. DIFFERENTIATION OF MEMORY T CELLS INTO EFFECTOR FUNCTIONS

Although the maintenance of memory and memory-cell division may not depend on restricted peptide recognition, it is very clear that antigen recognition greatly increases the rate of memory-cell division. After antigen stimulation, antigen-specific T cells proliferate much more than activated bystander T cells (Tanchot *et al*. 1997) leading to their gradual selection in the course of immune responses.

It is not yet known if antigen recognition has only a quantitative effect on cell division or if it is also required to induce certain effector functions. This may be difficult to determine, as bystander activation and antigen-specific responses frequently coexist, and antigen-specific and non-specific memory cells share similar early activation markers. Besides, it is not evident which T-cell activities may be considered effector functions, or how to identify effector T cells *ex vivo*. Lymphokine secretion can be considered as a T-cell effector function, but this is likely to occur even during bystander activation, as autocrine lymphokine production must be required to ensure memory T-cell division. Indeed $CD8^+CD44^+$ memory cells, but not $CD8^+CD44^-$ naive cells expressed mRNA coding for IL2 and INFγ perforin and Fas-L, in the absence of antigen (Tanchot *et al*. 1997). It remains to be established if antigen stimulation has an important influence on the amounts of lymphokines produced by memory T cells, and if other T-cell functions, such as T-cell cytotoxicity and the CD40L expression required to mediate T–B cell collaboration are induced only after cognate interactions.

To summarize, memory cells express an array of lymphokine receptors, costimulatory and adhesion molecules that probably modify the way they perceive their environment in ways that are not yet well defined. These stimuli may be triggered independently, ensuring the maintenance of restricted TcR specificity by memory T cells. Alternatively, they may reflect the overall T-cell avidity, increasing the number of peptides able to engage the memory TcR, and making T-cell recognition unstable and dependent on the degree of T-cell activation. The impact of these different possibilities will depend on the way in which different stimuli are integrated to generate different memory functions. Memory T-cell behaviour may result from an addition of different stimuli. Alternatively, a set of particular stimuli (e.g. TcR engagement) may be required to induce particular T-cell effector functions.

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